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LOW GENETIC DIVERSITY IN *Wolbachia*-INFECTED *Culex quinquefasciatus* (DIPTERA: CULICIDAE) FROM BRAZIL AND ARGENTINA

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SUMMARY

Culex quinquefasciatus is a vector of human pathogens, including filarial nematodes and several viruses. Although its epidemiological relevance is known to vary across geographical regions, an understanding of its population genetic structure is still incipient. In light of this, we evaluated the genetic diversity of *Cx. quinquefasciatus* and *Cx. pipiens* x *Cx. quinquefasciatus* hybrids collected from nine localities in Brazil and one site in Argentina. We used mitochondrial genes *cox1* and *nd4*, along with the *coxA* and *wsp* genes of the maternally-inherited *Wolbachia* endosymbiont. The *nd4* fragment was invariant between samples, whilst *cox1* exhibited four haplotypes that separated two types of *Cx. quinquefasciatus*, one clustered in southern Brazil. Low sequence diversity was generally observed, being discussed. Both Brazilian and Argentinian mosquitoes were infected with a single *Wolbachia* strain. As reported in previous studies with these populations, *cox1* and *nd4* diversity is not congruent with the population structure revealed by nuclear markers or alar morphology. Future *Cx. quinquefasciatus* research should, if possible, evaluate mtDNA diversity in light of other markers.

KEYWORDS: *Culex quinquefasciatus;* Genetic diversity; Mitochondrial markers; *Wolbachia*.

INTRODUCTION

Although *Culex quinquefasciatus* Say (Diptera: Culicidae) is a vector of human pathogens, including agents for filariasis and several arboviruses, its epidemiological importance varies considerably among regions[14]. In equatorial-tropical regions, the species is reported as the primary vector of *Wuchereria bancrofti* in the transmission cycle of lymphatic filariasis[36]. In subtropical and temperate urban areas, it is implicated as the primary vector of West Nile Virus[23] and other arboviruses[11].

Species of the *Culex pipiens* complex share morphological similarities and generally proliferate in human settlements, with *Cx. quinquefasciatus* adapted to tropical and subtropical areas and *Cx. pipiens* to temperate regions. Their ranges overlap in intermediate areas, resulting in genetic introgression and hybridization[10]. In Brazil, *Cx. quinquefasciatus* has an expansive distribution, including in almost all major cities[28]. Hybrids of *quinquefasciatus*/*pipiens* occur in Uruguay and central Argentina, whilst *Cx. pipiens* occupies regions southward into Argentina[4,30].

Because they are vectors of both urban and rural diseases, members of the *pipiens* subgroup have been targets of population control programs worldwide, with anthropophilic species killed by contact with chemical or biological reagents[19]. However, these populations often adapt resistance to these measures, with the selected organisms eventually expanding into different biogeographic regions.

In addition to pressure from control initiatives, these mosquitoes are often infected by endosymbionts such as *Wolbachia pipientis* rickettsies, which are associated with cytoplasmatic incompatibility (CI)[3]. Under CI dynamics, crosses between infected males with non-infected females produce eggs with decreased viability. Progenies originating from infected females and either infected or non-infected males are normally fertile. However, research has shown that offspring of individuals infected with different *Wolbachia* strains may be infertile[24]. Consequently, infected females have a reproductive advantage that leads to the expansion of infection in populations.

Recently, ALMEIDA[2] showed that infection by a single *Wolbachia* strain was ubiquitous in all *Cx. quinquefasciatus* samples tested from São Paulo City, Brazil. Among the *pipiens* subgroup, insecticide-resistant populations tend to be infected with higher *Wolbachia* densities[12]. It has been posited that a low efficiency of *Wolbachia* control may be related to mosquito immunity factors. In view of this, the manipulation of *Wolbachia* infections has generated enthusiasm in the field of vector control[21].

Despite its epidemiological relevance, population genetics data for *Cx. quinquefasciatus* are still sparse. We thus sought to analyze

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the genetic diversity of *Cx. quinquefasciatus* populations from urban areas of Brazil and central Argentina using cytochrome *c* oxidase subunit I (*cox1*) and NADH dehydrogenase subunit 4 (*nd4*) and these populations' patterns of *Wolbachia pipientis* infection through analysis of the *Wolbachia* surface protein (*wsp*) and cytochrome *c* oxidase subunit I (*coxA*).

MATERIALS AND METHODS

Mosquitoes. Adult mosquitoes were collected by aspiration[32] near residual-water channels during February and March 2008 in the Brazilian municipalities of Teresina (5°S), Recife (8°S), Rio Branco (9°S), Pariquera-Açu (24°S), Pelotas (31°S), Chapecó (27°S), Pontes e Lacerda (15°S), Santa Vitória do Palmar (33°S) and São Paulo (23°S), and in the Argentinian city of La Plata (34°S) (Fig. 1). Samples were stored in individual tubes on silica gel until processing. Thirty specimens from each locality (15 males and 15 females) were identified following taxonomic keys[15] and subsequently analyzed genetically.

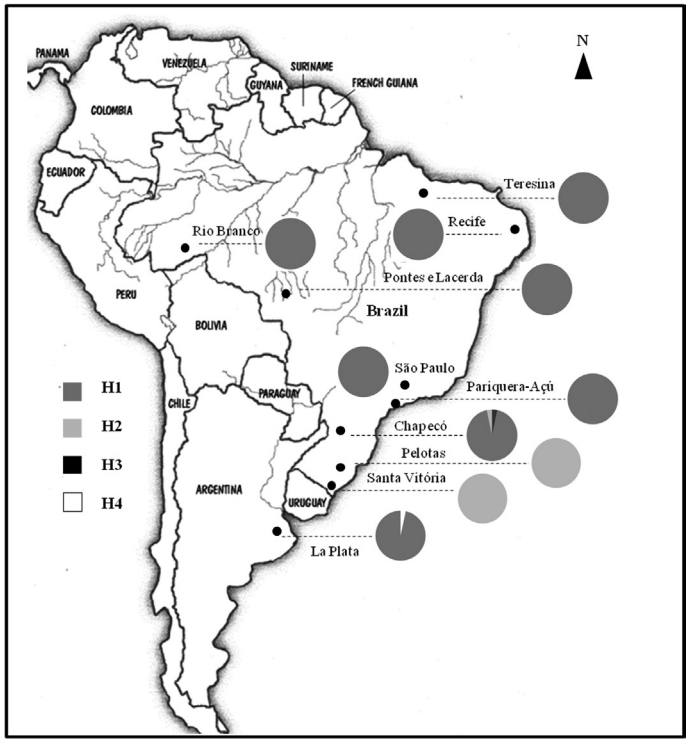

Fig. 1 - Map of South America with mosquito collection sites and distribution of four *cox1* haplotypes in Brazil and Argentina.

***DNA extraction and amplification of mitochondrial genes*.** Genomic DNA was isolated from individual mosquitoes using DNeasy® Blood & Tissue kits (Qiagen) following the manufacturer's instructions. DNA was eluted to a final volume of 100 µL and stored at -20 °C until polymerase chain reaction (PCR). Internal primers were designed to shotgun-amplify the complete 1260-bp *cox1* amplicon: two smaller fragments (~700-bp) were assembled following amplification using primers pairs Fly10[27] + coxF1 (5'-TTT GAG CTC ATC ATA TAT TTA-3') and UEA3[41] + coxR2 (5'-GCT CGT GTA TCA ACA TCT-3'). Primers ND4+/ND4-[16] were used to amplify the *nd4* gene.

PCR was performed in a final volume of 50 µL, which included 2 mM MgCl2, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.5 mM of each primer, 0.2 mM dNTP mix, 1U Taq DNA polymerase (Invitrogen), and 5-10 ng genomic DNA. The thermocycler program was configured for an initial denaturation step at 94 °C five min, followed by 35 amplification cycles (94 °C/60 s, 50 °C/60 s, and 72 °C/60 s) and a final elongation at 72 °C for 10 min.

Amplification of Wolbachia genes (*wsp *and* coxA*). Mosquito samples from Teresina, Rio Branco, Santa Vitória and La Plata were tested for *Wolbachia* using the total DNA preparations employed for PCR above. The PCR conditions described in ZHOU *et al.*[42] and SANOGO *et al.*[38] were used to amplify *wsp*. Samples from each mosquito population were then submitted to PCR-amplification of bacterial cytochrome *c* oxidase (*coxA*) to detect population/strain polymorphism using primers and conditions described in BALDO *et al.*[6].

DNA sequencing and sequence analyses. Amplified fragments were sequenced using BigDye TM Terminator 3.1 (Applied Biosystems), following the manufacturer's recommendations and directly sequenced on an ABI prism®3100. Sequences were analyzed using Chromas (Technelysium Pty), aligned with Clustalw2 (EBI) and edited with the software Geneious Pro 5.5.7 (Biomatters) and BioEdit 7.0.9 (Ibis Biosciences). Structural fragments were evaluated using ORF Finder (NCBI) and protein-structure predictions were developed initially through PSIPRED and then queried in the InterPro databases (NCBI). Pairwise, similarity data and genetic distance were calculated with Geneious Pro 5.5.7. Statistical data, such as haplotypes diversity (Hd) and nucleotides diversity per site (π) were tabulated from DnaSP v5.

RESULTS

Mosquito samples. *Culex quinquefasciatus* were caught almost exclusively in each mosquito collection. Samples were killed and preliminarily identified in the field, stored in individual tubes with silica gel (100 adult females and 100 adult males) and transported to the laboratory of the *Faculdade de Saúde Pública USP*, in São Paulo. At least two adult male and female vouchers from each locality were mounted and deposited in the *Coleção de Referência da Faculdade de Saúde Pública USP*, under access numbers E-13706 to E-13729. An equal number of females and males (N = 30) from each locality were separated for molecular analyses.

Mitochondrial molecular markers. Amplification of the *nd4* gene produced fragments of 321-bp (after primer trimming) (GenBank accession number GQ255653). This fragment showed high A+T content (72.6%) and 100% sequence identity among samples. The 1260-bp *cox1* fragment was comprised of four haplotypes (H1, H2, H3 and H4), whose geographical distribution is shown in Figure 1. The sequences were deposited in GenBank, under accession numbers: GQ255650 (H1), GQ255651 (H2), GQ255649 (H3) and GQ255648 (H4).

The H1 haplotype was that with the highest frequency (76.6%), followed by H2 (22.6%) and H3 and H4 (0.4%). Haplotypes H3 and H4 each contained one mutation in a single mosquito sample. To confirm the singleton status of these haplotypes, we repeated PCR and sequencing from the same genomic DNA. The alignment of the four haplotypes showed 99.7% genetic identity among geographic samples. Genetic

diversity of the *cox1* fragment was low: haplotype diversity (Hd = 0.636) and nucleotide diversity per site ($\pi = 0.00091$). The H2 haplotype was restricted to southern Brazil (Santa Vitória and Pelotas), but was also detected in a mosquito sample from Chapecó City.

Genic products. Prediction analyses confirmed the proteins cytochrome c oxidase 1 (COX1 EC1.9.3.1) and NADH dehydrogenase 4 (NADH4 EC1.6.5.3) as products of the *cox1* and *nd4* fragments, respectively. Analyses showed that the *cox1* transition (C→T) detected in a La Plata sample (H4) results in modification of the primary protein structure throughout the domain region. This amino acid modification did not show identity with other *cox1* enzymes. Other transitions in haplotypes H1, H2 and H3 did not produce changes in the primary protein structure. Pairwise comparisons of *cox1* resulted in 100% identity with *Cx. pipiens* and *Cx. quinquefasciatus* from other regions of the world, indicating homology and conserved functional domain.

Wolbachia infection. The *Wolbachia pipientis wsp* and *coxA* amplicon sizes were 504-bp (61% A+T) and 478-bp (62.6% A+T), respectively. All sequences for both genes were identical in all samples analyzed and have been deposited under GenBank under accession numbers HM563687 (*wsp*) and HM563686 (*coxA*). In comparison to the *Wolbachia* genome available from GenBank (accession number AM999887), the *wsp* sequence was 100% similar to the homologous region in the *Wolbachia wPip* strain, an endosymbiont of *Cx. quinquefasciatus.* The *coxA* sequence was also 100% similar to the homologous region of cytochrome *c* oxidase subunit 1 of the *wPip* strain.

DISCUSSION

Comparative analyzes with the partial *nd4* of *Cx. quinquefasciatus* showed 100% identity with mosquitoes from South Africa (AY793692) and Thailand (AY793692), and a one-base substitution in comparison to sequences from Riverside USA (AY793693). Although the complete *Cx. quinquefasciatus nd4* sequence is 1343-bp long (HQ724617), studies commonly use the primer pair used here (*nd4*+/*nd4*-) to produce an amplicon of approximately 350-bp located in the gene's central portion. This region has been widely used in others culicid mosquitoes in studies of genetic diversity, systematics and phylogeny, being that the *Cx. quinquefasciatus nd4* sequences yielded diversity indices higher for both *Anopheles*[31] and *Aedes*[26] genera (Hd = 0.895; $\pi = 0.0127$). The low variation seen in the *nd4* gene highlights its conservation and tendency toward homoplasmy in *Cx. quinquefasciatus*.

The variation seen in the four *Cx. quinquefasciatus cox1* haplotypes is incongruent with the results from the *ace2* intron and for mosquitoes of hybrid origin (with *Cx. pipiens*) found by MORAIS *et al.*[30], potentially because *ace2* mutates slower than *cox1*[20]. This suggests that mitochondrial DNA does not distinguish taxa within the *pipiens* subgroup. Sequences of *cox1* show both intra and inter-specific polymorphism, indicating that taxonomic diagnoses should include nuclear markers to avoid overestimates of diversity in *Cx. quinquefasciatus-pipiens* populations. However, mitochondrial markers identified different *Cx. quinquefasciatus* genetic types, including the Brazilian groupings.

The H1 haplotype is broadly distributed throughout the Brazilian tropics and in La Plata. It shares identity with the *Cx. quinquefasciatus* of tropical India (GenBank acc. no. FN395201). The H2 haplotype forms a population group, with a latitudinal range between 31 and 33°S in Brazil. It shares identity with *Cx. pipiens cox1* sequences from Ohio (DQ360492), *Cx. pipiens* (FN395187) and *Cx. p. f. molestus* (FN395179) from Russia, *Cx. p. pallens* (FN395203) from Japan and *Cx. quinquefasciatus* (DQ181446) from Puerto Rico.

The H2 haplotype is found predominantly in southern Brazil. Its restriction to this region may be a consequence of founder effects. The low frequency of H2 in Chapecó suggests that this city may represent the limits of haplotype's northern distribution. However, the La Plata population, which is genetically confirmed as a hybrid[30], contains individuals sharing *nd4* and *cox1* genes with pure *Cx. quinquefasciatus* elsewhere. We suggest that reproductive isolation is incomplete within the *pipiens* subgroup and that this affects the taxonomic resolution usually provided by mitochondrial genes, including gene *cox1* barcode.

The differences between haplotypes H3 and H4 are insufficient to ascertain their relevance to intra-specific divergence or their relationship with mosquitoes from other parts of the world. Single mutations were also found in *cox1* sequences by COOK *et al.*[9] and reflect the polymorphic character of this gene.

Prediction tests showed that the four-base substitutions detected in *cox1* are transitions. Three of these are synonymous and two form population groupings. Transitions and synonymous substitutions were also found by NAVAJAS *et al.*[33] in other insects *cox1*. According to those authors, changes in the base composition of mitochondrial genes occur most frequently through synonymous transitions, without detectable effects on gene functioning. However, the sequences enabled the identification of divergence and gene flow dynamics in biogeographically distinct populations.

The low diversity of *Cx. quinquefasciatus* and *Cx. pipiens* mitochondrial genes has been reported by GUILLEMAUD *et al.*[17]. HASAN *et al.*[18] also found low diversity (Hd = 0.502 and $\pi = 0.0007$) in the *Cx. quinquefasciatus cox2* of populations from central Bangladesh, potentially a result of a recent common mitochondrial ancestor. Such data may also be explained by the recent and rapid expansion of these species (assisted by human migration and population growth) and successive bottlenecks caused by control measures in urban areas[13].

Culex quinquefasciatus populations have been significantly impacted by control programs in Brazil, particularly in northern and northeastern equatorial regions, where the species is the primary vector of lymphatic filariasis and dirofilariasis[1]. The regions' low altitude, hot and humid climate and constant thermal amplitude facilitate both the development of the heartworm pathogen and the hematophagy of *Culex* vectors throughout the year[25].

Unlike, vector control programs are less intense in southern Brazil due to the humid subtropical climate and high peaks of thermal amplitude. Although *Culex* populations in these areas have shown resistance to thermal shock, they suffer reduced numbers of over-wintering individuals and lower metabolic activity during cold months[37]. Although no cases of heartworm have been identified from southern Brazil, there are records of encephalitis and arbovirosis[8]. In Argentina, members of the *pipiens* complex are implicated as vectors of arboviruses, such as Saint Louis Encephalitis[11].

Due to the epidemiological importance of *Culex*, vector-control programs have been organized in urban and rural areas for decades. These have involved countless chemical insecticides such as pyrethroids, Dichloro-diphenyltrichloroethane (DDT) and their derivades[5]. Organophosphates, *Bacillus thuringiensis*[7] and *Bacillus sphaericus*[29] larvicides have also been used. The exposure of mosquito populations to insecticides often confers resistance[40] and the expansion of resistant individuals can explain population-level selective sweeps[34].

On another view, Brazilian *Cx. quinquefasciatus* populations share identical *nd4* sequences to those of RASGON *et al.*[35] in North America (GenBank acc. no. AY793688) and parts of Asia (AY793691), but are from 5 to 6% divergent from South African populations (AY793694). These authors suggest that mitochondrial diversity may be related to *Wolbachia* infection: the American and Asian samples, which are infected, have low diversity, whilst the uninfected populations of South Africa have higher mitochondrial variation. All *Culex* samples are apparently infected by a single *Wolbachia* strain because of the invariant *wsp* and *coxA* sequences. This appears to be *wPip*, with which the *Wolbachia* infections herein share sequence similarity and similar hosts: *Cx. quinquefasciatus* and *Cx. pipiens*.

The fact that, despite being geographically scattered, all populations sampled herein share the same *Wolbachia* strain may be explained by host-endosymbiont specificity, as pointed out by WERREN *et al.*[39]. The genetic homogeneity observed among *Wolbachia* samples may also indicate that this endosymbiont only recently infected these *Culex* populations.

Culex quinquefasciatus appears to possess two mitochondrial types in Brazil. This fact should be taken into consideration in investigations of disease distribution and in aspects of blood-hosts in those locations. Recent studies suggest that feeding preferences may be influenced by genetic factors[22].

RESUMO

Baixa diversidade genética em *Culex quinquefasciatus* (Diptera: Culicidae) infectado por *Wolbachia* do Brasil e Argentina

Culex quinquefasciatus é vetor de patógenos humanos, incluindo nematódeos filarídeos e vários vírus. Embora a sua relevância epidemiológica varie entre as diferentes regiões geográficas, o conhecimento da estrutura genética da população é ainda incipiente. Em vista disso, foram avaliados os níveis de diversidade genética de *Cx. quinquefasciatus* e de híbridos *Cx. quinquefasciatus* x *Cx. pipiens* de nove cidades do Brasil e em La Plata, na Argentina. Para os testes foram utilizados fragmentos dos genes mitocondriais *cox1* e *nd4*, juntamente com *coxA* e *wsp* do endossimbionte *Wolbachia*, herdado maternalmente. O fragmento *nd4* não apresentou variação entre as amostras, e o *cox1* exibiu quatro haplótipos que separaram dois tipos de *Cx. quinquefasciatus*, com um deles agrupado no sul do Brasil. Os dados de sequência mostraram baixa diversidade, sendo esta discutida. Ambas as amostras de mosquitos brasileiros e argentinos estão infectados com uma única cepa de *Wolbachia*. A diversidade apresentada por *nd4* e *cox1* não é congruente com a estrutura da população revelada por marcadores nucleares e morfologia alar de estudos anteriores com estas mesmas populações. Pesquisas com *Cx. quinquefasciatus* devem, se possível, avaliar a diversidade por DNA mitocondrial na luz de outros marcadores.

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